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Local 5-Aminolevulinic Acid Application for Laser Light–Induced Fluorescence Diagnosis of Early Staged Colon Cancer in Rats

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Background and Objectives: **5-Aminolevulinic acid (ALA) increases the intracellular accumulation of endogenous protoporphyrin IX in colon cancer. Protoporphyrin IX itself is a potential photosensitizer that can be used for laser light-induced fluorescence diagnosis. The aim of this study was to detect cancer in the rat colon before macroscopic visibility.**
Study Design/Materials and Methods: **Multifocal colon carcinomas were induced by weekly subcutaneous injections of 1,2-dimethylhydrazin-dihydrochloride in male Wistar rats. Local photosensitization was performed with an ALA colon lavage. Red fluorescence (635 nm) was induced by green laser-light irradiation with an Ar-Dye Laser (514 nm) in the colon. Fluorescence was observed by the naked eye with a filter at < 515 nm to eliminate the excitation light.**
Results: **Twenty-five Wistar rats developed 99 macroscopically visible carcinomas and four macroscopically visible dysplasias. The following laser-light–induced fluorescence diagnosis procedure was able to detect 16 additional carcinomas and 41 additional dysplasias.**
Conclusions: **Local ALA application induces a tumor-specific protoporphyrin IX accumulation in the rat colon and is an efficient method for fluorescence detection of invisible dysplasias and early colon cancer in the rat. Lasers Surg. Med. 26:302–307, 2000.** **© 2000 Wiley-Liss, Inc.**

Key words: colon carcinoma; fluorescence diagnostics; ALA; aminolevulinic acid; ulcerative colitis; endoscopy

INTRODUCTION

Dysplasia and early cancer of the colon may not always be diagnosed by endoscopy. Small colonic neoplasms are frequently overlooked during colonoscopy, whereas other tumors are completely occult to the endoscopist. Random biopsies solve this problem only insufficiently. It is further complicated in inflammatory mucosa such as ulcerative colitis. On the other hand, early detection of colon cancer or premalignant dysplasias improves survival and prognosis of the patients [1].

It has already been demonstrated that laser-light–induced fluorescence after systematic photosensitization is able to differentiate between normal and malignant colon mucosa [2–4]. Usually, haematoporphyrin derivatives such as Photofrin II© were used for photosensitization of malignant gastrointestinal tumors. The primary side effect of systemically applied photosensitizer (PS) is the prolonged photosensitivity of the entire body with a high risk of heavy sunburns after daylight exposure for weeks. The duration of photosensitivity can be reduced to 24 hours by oral application of aminolevulinic acid (ALA) [5]. ALA is a natural precursor of heme and the first committed intermediate in the heme biosynthesis pathway. The excess of systemically nonphotoac-

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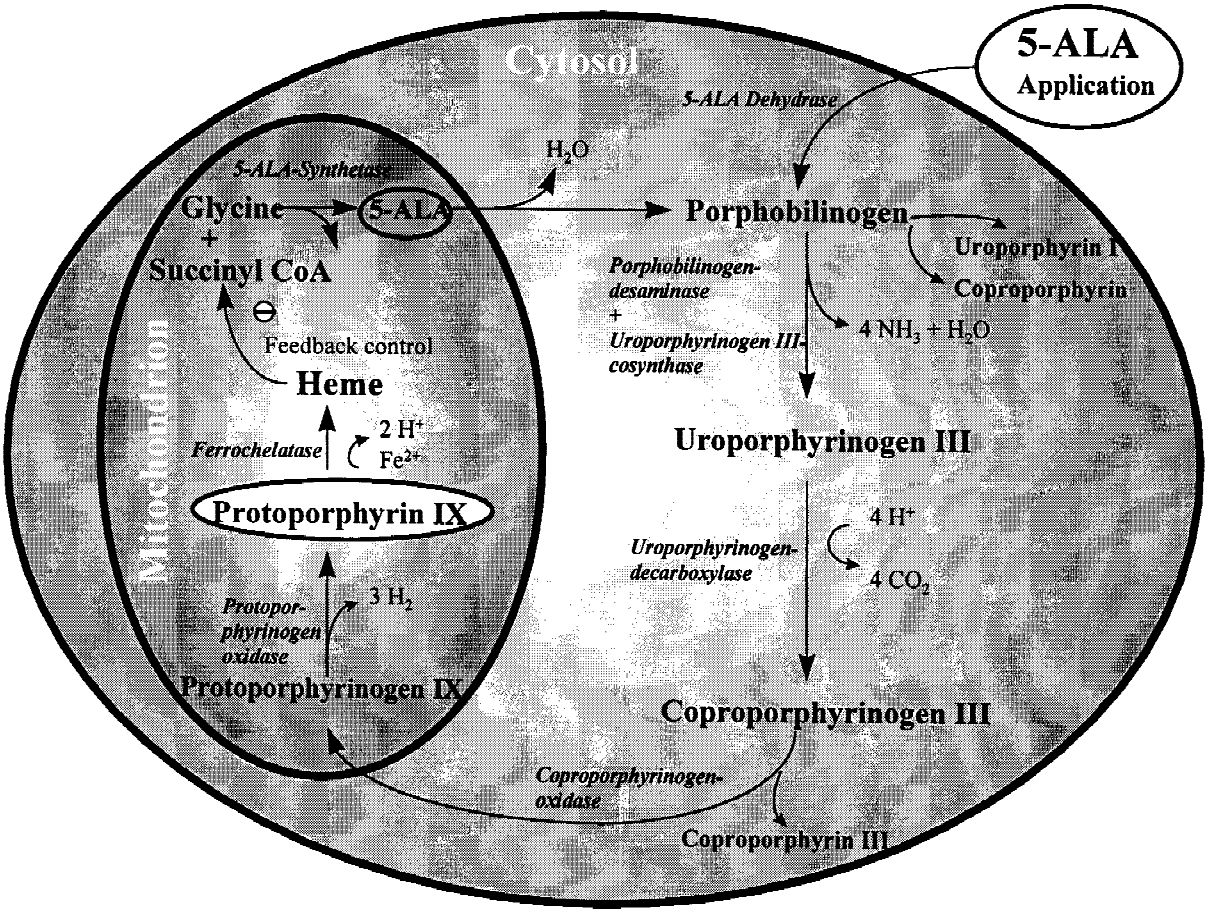

Fig. 1. Heme biosynthesis and aminolevulinic acid (ALA) metabolism into protoporphyrin IX (PpIX). CoA, coenzyme A.

tive ALA causes an accumulation of photoactive protoporphyrin IX (PpIX), the last metabolite before heme synthesis, by overloading the regulatory mechanisms (Fig. 1). PpIX itself is a photoactive agent with an absorption spectrum typical of porphyrin. Activation with green light at 514 nm causes an observable fluorescence within the red band at 635 nm. Fluorescence kinetic studies showed maximal PpIX accumulation in gastrointestinal tumors 4 hours after oral and local application [5]. This time seemed to be the optimal interval between ALA administration and light treatment (photodynamic therapy, PDT). Another advantage of ALA-induced endogenous porphyrin biosynthesis is the variability of possible administration. Apart from intravenous and oral application, promising results have been obtained in PDT and laser-light–induced fluorescence diagnosis (LIFD) with topical and local application like inhalation or hollow organ filling [6,7]. Intravesical instillation of ALA induces red porphyrin fluorescence for cystoscopic detection of early bladder cancer. The intravesical ALA-induced porphyrin fluorescence procedure shows a sensitivity of 94.2% and specificity of 80.0% in a clinical trial [6]. A protoporphyrin IX (PpIX) accumulation in gastrointestinal cancer has already been proven in many experimental tumor models and used clinically for endoscopic photodynamic therapy (PDT) [8,9]. In a dimethylhydrazin (DMH) rat colon carcinoma tumor model, it has already been shown that intravenous application of ALA induces the tumor-specific intracellular PpIX synthesis with sufficient differentiation between normal and malignant cells [10,11]. We studied the fluorescence detection of DMH-induced multifocal colon carcinomas in rats after intraluminal application of ALA aiming at the detection of yet invisible lesions in the rat colon.

MATERIALS AND METHODS

Tumor Model

Multifocal colon carcinomas at all stages were induced by weekly subcutaneous injections

TABLE 1. Number of Detected Colon Carcinoma and Dysplasia Cases Either Macroscopically or With Laser Light–Induced Fluorescence Diagnosis

Group	Carcinoma	Dysplasia	False-positive findings	Total no. of malignant and premalignant foci
A: Macroscopic inspection	99	4	3	103
B: Fluorescence positives of Group A	92 (92.9%)	4 (100%)		
C: Only fluorescence positive = additional detected	16	41	11	57
				160

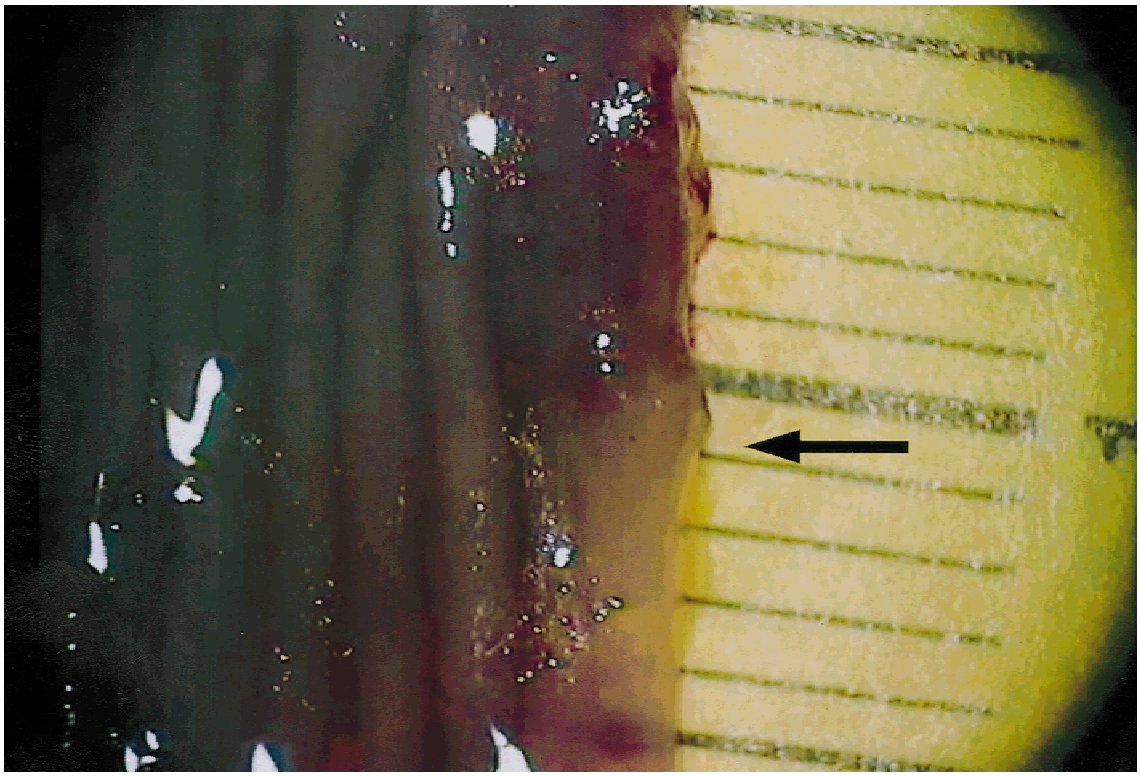

Fig. 2. Native and macroscopically unsuspicious rat colon mucosa after 17 weeks of dimethylhydrazin tumor induction.

of 1,2-dimethylhydrazin-dihydrochloride (sym DMH; M_r, 133.02; 21 mg/kg body weight [BW]) (Medac, Germany) over 21 weeks in male Wistar rats as reported by Druckrey et al [12].

The protocol was approved by the office for animal experiments (Regierungspräsidium Karlsruhe, Germany). The animals were at an age of 4–6 weeks and their BW ranged from 200 to 250 g. They were kept under standard laboratory conditions with free access to water and standard food. BW increased to the range 500–600 g after 21 weeks of DMH treatment.

Photosensitization and Fluorescence Imaging

After 21 weeks of tumor induction, the animals were anaesthetized with Ketanest©/ Dormicum© and underwent a small median laparotomy in the lower abdomen with mobilisation of the coecum. After a small coecal incision, a probe was placed into the lumen and fixed with a loop suture. Subsequently, the terminal ileum was ligated. Stools were washed out by a colon lavage through the probe with 20 ml of sterile 0.9% isoton NaCl solution until the lavat became clear. The complete NaCl solution was then replaced by 25 ml of a sterile ALA solution (0.17 M $Na^+HCO_3^-$; ph 6.5, Sigma, St. Louis, MO), and the anus was closed with a running loop suture.

Four hours after incubation, a total colectomy was performed and the explanted colon was cut open longitudinally and pinned on a cork plate. Tumor foci visible by white light were first

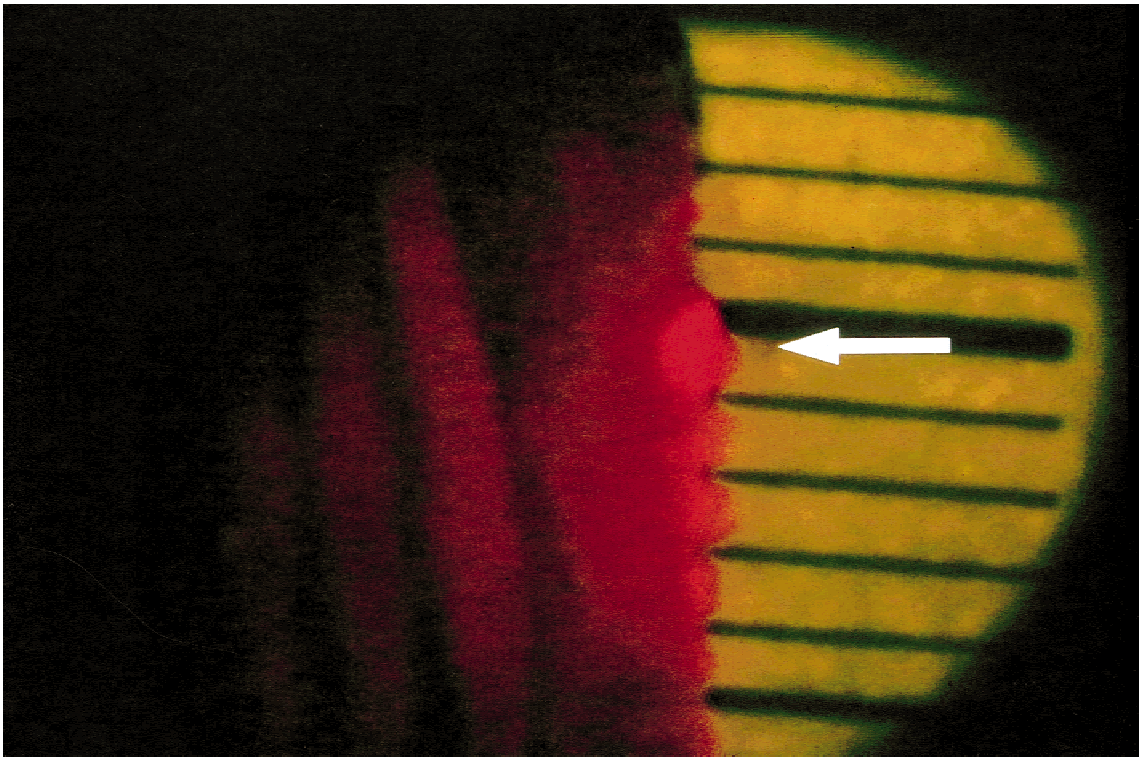

Fig. 3. Ar-dye laser irradiation (514 nm) of the same rat colon section as in Figure 2. Fluorescence imaging with a <514-nm filter and detection of a red fluorescent colon carcinoma (arrow). Histopathologic examination (hematoxylin and eosin staining) showed nuclear stratification, pleomorphism, hyperchromatism, and loss of polarity with extension through the muscularis mucosae.

counted and mapped; subsequently, red fluorescence (635 nm) was induced by green laser light irradiation with an Ar-Dye Laser (Spectra Physics, Germany) at 514 nm wavelength and a bare fibre. Fluorescence was observed by the naked eye with a filter at < 515 nm to eliminate the excitation light. Visible fluorescence spots (red, 635 nm) were marked with tissue colour (Davidson Tissue Marking System, Bradley Products, Inc., Bloomington, MN). Hematoxylin and eosin (HE) stainings of each tumor were performed to verify the colon carcinoma or dysplasia.

Histology

All identified colonic lesions were analysed microscopically after HE staining and classified as dysplasia or carcinoma. All neoplastic epithelial proliferations characterised by nuclear stratification, pleomorphism, hyperchromatism, and loss of polarity without invasion of the lamina propria were classified as dysplasia. Invasion of the lamina propria by cytologically malignant epithelium or extension through the muscularis mucosae was classified as colon carcinoma. To avoid pathologist-depending high interobserver variabilities resulting in uncontrolled errors, dyspalsias were not graded [13,14].

RESULTS

Twenty-five Wistar rats developed 99 macroscopically visible colon carcinomas and four macroscopically visible dysplasias. Thus, the total number of premalignant and malignant lesions macroscopically detectable was 103 (99 + 4 cases) (Table 1). All dysplasias and carcinomas in this group were histologically verified by HE stainings. However, macroscopic inspection showed three visible tumors as false positive upon HE staining and histologic examination. During laser light irradiation, 92 (92.9%) of the macroscopically visible colon cancers showed red fluorescence and were fluorescence positive. All four macroscopically visible dysplasias were also fluorescence positive (100%). The following LIFD procedure showed 68 additional red fluorescence spots undetectable for the naked eye (Figs. 2, 3). Histologic investigation (HE staining) showed

that LIFD enabled us to detect additional carcinomas and additional dysplasias: 16 carcinomas and 41 dysplasias were only identified by laser-induced red fluorescence (Table 1). Consequently, a total number of 57 detected malignant and premalignant colon lesions could be diagnosed in this tumor model. A total number of 11 fluorescence positive foci showed no evidence for malignancy in the histopathologic workup (HE staining) and were count as false positive (Table 1).

For a total number of 160 (103 + 57) detected malignant and premalignant tumors the calculated sensitivity for macroscopic inspection is 64% (103/103 + 57) and reaches 96% (92 + 4 + 16 + 41/103 + 57) for the LIFD procedure (Table 1). The positive predictive value is 97% (103/103 + 3) for macroscopic inspection and 93% (92 + 4 +16 + 41/153 + 11) for the LIFD procedure (Table 1).

DISCUSSION

Early stage cancer and dysplasias are difficult to identify by colonoscopy. Small lesions may be missed and macroscopically invisible tumors and epithelial dysplasias are not detectable. Multiple or random biopsies sample a very small amount of the mucosal area of the colon and represent less than 0.1% of the surface (sampling error). Repeatability of biopsy sampling of suspicious lesions during colonoscopies in annual surveillance programs is a problem, because it is nearly impossible to take biopsy specimens from the same mucosal site (sampling error). Considering the inefficiency of multiple biopsies for detecting cancer in the colon, malignancies occurring in the interval between two examinations are more likely to be cancers missed in the previous screening (false-negative results) [15].

In patients with ulcerative colitis and a high risk of colorectal cancer, carcinomas and dysplasias may be missed despite surveillance programs and random biopsies. Fifty-three (8.3%) patients with ulcerative colitis developed a colitis-associated colorectal carcinoma in a clinical series of 643 patients [16]. A total of 22.6% of these colorectal carcinomas were not diagnosed in previous colonoscopies, including the preoperative endoscopy. In addition, 12.2% of the patients with cancer showed multifocal colorectal lesions diagnosed in the resected specimen only. There are no data available on the amount of undetected lesions, but it must be assumed that there are many more undetected early cancers or dysplasias in high-risk patients [16]. On the other hand, the prognosis of colorectal cancer depends on the tumor staging and early detection especially in high-risk patients is desirable [1].

LIFD after ALA colon lavage is a suitable method for detection of macroscopically invisible colon cancer and dysplasias in the DMH-induced rat colon carcinoma model. Apparently, the only fluorescence-positive dysplasias (n = 41) and colon carcinomas (n = 16) would elude conventional diagnostic methods like colonoscopy or even open examination through the pathologist. Based on the high rate of additional detected dysplasias in the rat colon all macroscopically visible dysplasias (n = 4) were fluorescent positive. However, it must be mentioned that there were 11 false-positive fluorescence spots during LIFD procedure in this tumor model. Histology confirmed these spots as normal mucosa. LIFD seems to be a helpful method to detect additional colon dysplasias with a predictive value of 93%. There were even manifest colon carcinomas (n = 16), which were not detectable by the naked eye.

The aim of the study was to detect malignant and premalignant tumors of the colon before macroscopic detectability. However, all macroscopically visible dysplasias and 92.9% of the macroscopic carcinomas were fluorescence positive. Thus, it seems possible to increase sensitivity of colonoscopy by a better visualization of malignant and premalignant colon tumors that are difficult to identify.

PpIX accumulation after systemic ALA application has already been proven in clinical situations of colon cancer and used for PDT and PDD [2,8]. It may be assumed that clinical intraluminal ALA application into the colon (enema, installation by means of colonoscopy) will lead to tumor-specific PpIX accumulation with efficient fluorescence labeling of colon cancer and dysplasias.

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